

Rapid Method For Transmission Electron Microscope Study Of *Staphylococcus aureus* ATCC 25923

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ABSTRACT

Technique development plays an important role in the field of electron microscopy. In this study, the Rapid Method for Transmission Electron Microscopy (RMTEM) was compared the Standard Method for Transmission Electron Microscopy (SMTM). By doing this we expect that the RMTEM to be the better choice and would give the electron microscopists a better understanding of the ultra structure of the *Staphylococcus aureus* ATCC 25923. The bacteria sample preparation showed that RMTEM had significantly higher mean grey values as compared to the SMTM ($t(58) = 6.614$, $p < 0.001$; CI95 = 321.329, 600.271). This demonstrates that the contrast produced for RMTEM is lower than the SMTM. This is due to the inability for bacterial protein and lipid constituents to reorganize into a more compact configuration. Furthermore, the RMTEM would cause less extraction of protein and lipid constituents compared to the SMTM. This was proven by the thickness of the mean outer membrane of the bacteria that were processed by the RMTEM compared to the SMTM ($t(58) = 8.460$, $p < 0.001$; CI95 = 7.2853, 11.8014). However, the mean cross sectional area of the bacteria is significantly reduced in the RMTEM compared to the SMTM ($t(58) = -4.202$, $p < 0.001$; CI95 = -40078.459, -14215.007). The later finding indirectly shows that the SMTM would cause damages to the bacteria's outer membrane and the plasma membrane causing an increase in bacteria cell size. This could be due to the movement of water molecules from the environment to the intracellular compartment of the bacteria, caused by the protein and lipid extraction from the outer membrane and plasma membrane of the bacteria. With such properties, the RMTEM would be the better choice in providing better detailed images for the ultra structures of the bacteria compare to the SMTM.

INTRODUCTION

The detailed understanding and characteristic of the cell structure often requires analysis by electron microscopy. Light microscopy offers limited analysis and is often too broad in resolution to distinguish the intimate subcellular structures. Studies of biomembranes and membranous organelles require transmission electron microscope, as the resolving power of light microscopes is often insufficient to analyze subtle changes in topological and structural details (Christoph *et al.*, 2001).

Resolution in the electron beam is normally limited by contrast and not of its resolving power. Contrast of images is determined by the nature and extent of interactions between the electron beam and the specimen. Contrast is defined as the relative difference in intensity between an image point and its surroundings. Contrast can also be defined as the relative difference to the grey value of the image. This grey value is sometimes referred to as the intensity of a pixel of the image. The amount of contrast that occurs at any particular specimen point is dependant on

the specimen's density and overall thickness and is relatively independent of the atomic number, chemical composition or other specimen properties (Christoph *et al.*, 2001). Hence, as mass thickness increases, the probability of contrast increases. However, contrast may be increased by the preferential addition of materials of high atomic number during specimen preparation (Santhana *et al.*, 2006).

In this study, we chose the *Staphylococcus aureus* ATCC 25923 as our study subject because of the size and the generation time of the bacteria. The generation time of the *Staphylococcus aureus* are generally 20 minutes. In view of this, we fixed our study matter at 20 minutes for glutaraldehyde (GA) and 5 minutes for osmium tetroxide (OT). The idea was to confine the living process of the bacteria as close to the first generation and the same time maintains the overall structure of the bacteria. This was based on the observation in the light microscopy that the bacteria were active in the GA for 72 hours and OT for 1 hour. We concluded from the observation that the bacteria would be fixed but the living process of the bacteria will continue. Also by doing this, we expected that the contrast of the image of bacteria and the damage to the structure of the bacteria would be reduced. Another important factor of the study was the introduction of en bloc staining prior to the OT fixation. We expected that the uranyl acetate (UA) would block the OT from damaging the structure of the bacteria because the OT is a very strong oxidizing agent. All these features were included in RMTEM and would be compared to the SMTEM.

MATERIAL AND METHODS

Bacteria Culture

Staphylococcus aureus ATCC 25923 was obtained from Bacteriology Unit, Institute Medical for Research, Kuala Lumpur. The concentration of the bacteria that was used in this study is 1.4×10^7 bacteria per test and the ratio between the bacteria and the fixative solution was at 1:15.

RMTEM

The bacteria that were fixed for 20 minutes in the 2% GA in 0.1M PBS were washed with distilled water (X6). Then the bacteria were washed with distilled water (X3), after it were stained with 2% UA for 5 minutes. The excess OT was discarded after the stained bacteria were exposed to OT for 5 minutes. The dehydration of the exposed bacteria to OT was conducted to the series of acetone (50%, 70%, 90% and 100%X), respectively 5 minutes. Polymerization was done with pure epoxy resin in the embedding oven at 75°C/2 hours and 90°C/2 hours, after the bacteria infiltrated by mixture of acetone and epoxy resin (1: 1) for 15 minutes. The blocks were trimmed and cut to 90 nm ultra thin sections and mounted on 200 mesh thin bar copper grids (Agar). The specimens then were stained with Reynold's stain for 1 minute. Each specimen was examined at 30 000 magnification by using Technai G2 TEM at an accelerating voltage of 90 KV.

SMTEM

The bacteria that were fixed for overnight in the 2% GA in 0.1M Cacodylate buffer were washed with 0.1M Cacodylate buffer (X3), each 20 minutes. Then the bacteria were washed with 0.1M Cacodylate buffer (X3), respectively 20 minutes, after it were exposed to 2% OT in 0.1M Cacodylate buffer 1 hour. The dehydration of the exposed bacteria to OT was conducted to the series of ethanol (50%, 70%, 90% and 100%X), each 20 minutes respectively. Polymerization was done with pure epoxy resin in the embedding oven at 60°C/18 hours after the bacteria infiltrated by mixture of ethanol and epoxy resin (1: 1) for 1 hour. The blocks were trimmed and cut to 90 nm ultra thin sections and mounted on 200 mesh thin bar copper grids (Agar). The specimens then were stained with UA and Reynold's stain at 10 minutes respectively. Each specimen was examined at 30 000 magnification by using Technai G2 TEM at an accelerating voltage of 90 KV.

Measurements of the structures of the *Staphylococcus aureus* ATCC 25923

For the determination of effects of the RMTEM and SMTEM in the preparation of specimen for TEM was focused on the thickness of the outer membrane, the grey values and the area of the cross section of the bacteria. The above mentioned measurements were measured by the Megaview II Soft Imaging System software. The mean thickness of the outer membrane, the grey values and the area of the cross section of the bacteria of 30 bacteria each from the RMTEM and SMTEM batches respectively were analyzed by unpaired t statistical software SPSS.

RESULTS

The electron micrographs that were obtained for the RMTEM, clearly shows the definitive structures (i.e. plasma membrane, outer membrane, ribonucleus, and the nucleotide) of the *Staphylococcus aureus* ATCC 25923 compared to the SMTEM (Figure 1). In addition, the mean contrast of the images of the bacteria for the RMTEM was reduced compared to the images of the bacteria for the SMTEM ($t(58) = 6.614$, $p < 0.001$; $CI_{95} = 321.329, 600.271$). The darker areas were far more differentiated than the lighter areas in the RMTEM compared to the SMTEM. Moreover, the mean thickness of the outer membrane of the bacteria by the RMTEM was thicker compared to the bacteria by the SMTEM ($t(58) = 8.460$, $p < 0.001$; $CI_{95} = 7.2853, 11.8014$). However, the mean area of the cross section of the bacteria by the RMTEM was reduced compared to the cross section of the bacteria by the SMTEM ($t(58) = -4.202$, $p < 0.001$; $CI_{95} = -40078.459, -14215.007$). The biostatistics results were summarized in the Table 1. Moreover, the time of processing the bacteria was markedly reduced in the RMTEM as compared to the SMTEM, by as much as 75%.

DISCUSSION

The objective of this study was to optimize the contrast of the TEM protocol and the electron micrographs of the bacteria by the RMTEM proves that, by clearly shows the outer membrane, plasma membrane, ribonulcei and nucleotides compared to the images of bacteria by the SMTEM (Figure 1). The grey area of the bacteria from the RMTEM such as cell matrix in the cytoplasm, plasma membrane and the outer membrane were well differentiated. This observation indicates that the contrast of the images of the bacteria were reduced significantly in the RMTEM compared to the SMTEM. Moreover, the thickness of the outer membrane of the bacteria was thicker in the RMTEM compared to the SMTEM. We expected these events to take place in the RMTEM because of the reduced time of the GA exposure to the bacteria. With these observations, we believed that the GA did not extract the proteins of the bacteria and did not fix the bacteria as strongly as in the SMTEM.

The contribution of contrast to the images of the bacteria by using the SMTEM compared to the RMTEM were proven by the findings of the work done by Jeareanaikoon and Abraham (2005). They reported that the mean diameter of the fixed algae cells was smaller than the unfixed algae cells; the reasons would be that the uptake of GA by the cells would reorganize the proteins in more closely packed configuration and would increase the contrast of the image of the cells. In the addition to the closely packed configuration of proteins, Kanamura (1975) also reported that G6P enzyme (an protein constituent) would be extracted if the enzyme were exposed longer in the GA solution compared to none extraction when shorter fixation time was used. The other study that supports these findings will be the work done by Margineanu and Van Driessche (1990). They reported that interaction between the membranes of the epithelial cells of the skin of *Rana temporaria* was greater in the first 10 minutes and slowing down in the 60 minutes, until the characteristics of the two membranes could not be distinguished any more. However, both of the samples that

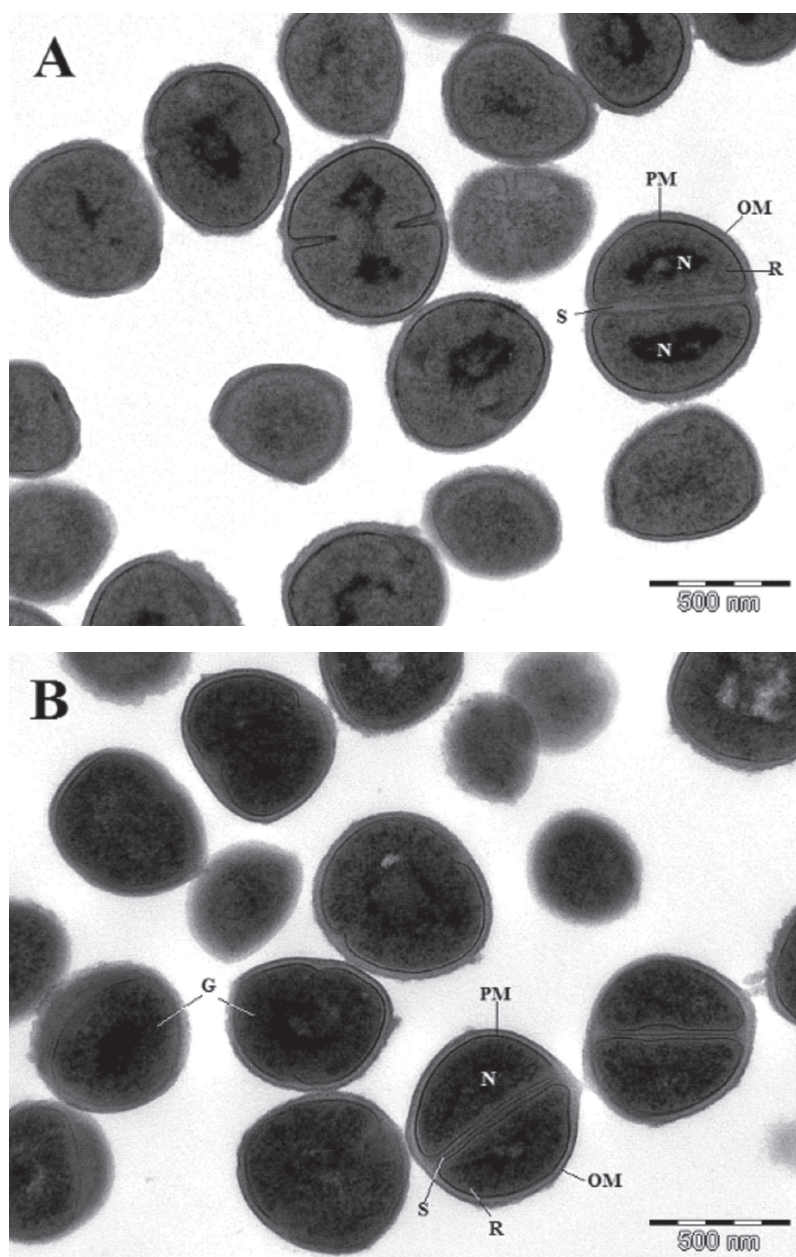


Figure 1. The electron micrograph of RMTEM (A) and SETEM (B). (A) shows very distinct OM=Outer Membrane, M=Plasma Membrane, R=Ribonucleus, N=Nucleotide, and S=Septa of the bacteria compare to (B). The granularities=G were prominent in (B) compare to (A). The magnification at 30 000X.

Table 1. Mean values of area of the thickness of the outer membrane, mean of grey values and the area of the cross section of the *Staphylococcus aureus* ATCC 25923.

<i>Staphylococcus aureus</i> ATCC 25923	RMTEM	SMTEM
Mean thickness of the outer membrane	29.6 nm	20.1
Mean grey values	1850	1389
Mean area of the cross section	252742 nm ²	279888 nm ²

were used in their studies are more complex and thicker compare to the bacteria that we used in this study. Therefore, the penetration and effects of the GA would be harsh for the bacteria, if they were exposed for a longer time as in the SMTEM. Even though the GA was exposed shorter time in the RMTEM, it maintained the overall structure of the bacteria (e.g. outer membrane, plasma membrane, and contents of cytoplasm) as indicated in the literature review of the bacteria.

Another factor contributing to contrast of the images of the bacteria would be the OT solution. The OT solution is generally used as the post fixative in TEM protocols. The benefits of OT would be the fixation of the lipids constituents and effects of its imparting of electrons to the sample. In view of these, we modified the standard preparation of OT by changing the diluent and duration of the exposure time. We expected that by doing this would reduce the contrast of the image of the bacteria (Figure 1). The results that we obtained were encouraging, based on the overall structure and detailed ultra structure of the bacteria. On previous scientific evidence, we noted that longer exposure by the OT would extract the proteins from the bacteria and fix the phospholipids of the bacteria very strongly. These evidences were noticed in the images of the bacteria by the SMTEM compared to the images of the bacteria by the RMTEM. Whereby, the outer membranes of the bacteria by the RMTEM were thicker and lightly colored compared to the bacteria by the SMTEM (Figure 1). The shorter time of fixation by OT, as in the RMTEM did not show any conjugated effects by the dehydrating solution such as the acetone. This proves that the GA weakly fixed the lipid constituents prior to the OT exposure.

Besides that, the bacteria in the SMTEM had more comprehensive imparting electron by OT because the cacodylate buffer reduced the water surface retention force that was present in the intracellular structures of the bacteria. By reducing the water surface retention, electron density around the phospholipids and the proteins were increased and contributed to the contrast of the images of the bacteria as in the SMTEM. However, in the RMTEM's bacteria this event of imparting electron by OT was reduced because of the distilled water was used as a diluent because the distilled water would not reduce the water surface tension very significantly as the cacodylate buffer did on the bacteria intracellular structures.

Another reason of reduced contrast by the OT in the images of the bacteria as in RMTEM would be the introduction of the UA properties before the bacteria were exposed to the OT solution. This was done because of the staining properties of the UA on the lipid constituents of the bacteria. By using this stain, the bacteria would be protected from increased imparting electrons of the OT solution. The decrease imparting electron effects were more evident in the bacteria by the RMTEM compared to the bacteria by the SMTEM. The mentioned effects were seen in the bacteria's' different grey areas from the outer membrane, whereby the contents phospholipids are more compared to the plasma membrane and the cytoplasm of the bacteria. This means that the imparting of electrons by the OT to the lipids were less and it was portrayed by the lightly colored outer membrane in the bacteria by the RMTEM compared to the bacteria by the SMTEM. The imparting of electrons of OT and its contributions were stated in the work done by the Sumner and Ross (1989) and supported our view in the shorter time in the fixation of osmium tetroxide that was used in the RMTEM.

Furthermore, this stain would also protect the bacteria from the damages by the OT solution. The damages of the bacteria were evident in the bacteria by the SMTEM compared to the bacteria by the RMTEM. As presented in Figure 1, the bacteria by the SMTEM have thinner outer membrane, patchy plasma membrane and granularities ribonucleic and this was due to the extraction of the protein constituent of the bacteria. We also believed that the extractions had cause the movement of water molecules from the environment into the bacteria. Even though, the 0.1M cacodylate buffer were used in the preparation of the OT in the SMTEM; the buffer were relatively a hypertonic state compared to the hypotonic environment in the intracellular of the bacteria. These movement water molecules had made the bacteria of the SMTEM to be bigger in

size compared to the bacteria of the RMTEM. The mean area of the cross section of the bacteria by the SMTEM supported these observations, which were increased by 33% compared to the mean area of the cross section bacteria by the RMTEM.

We also believed that the longer polymerization of the resin would contribute to the contrast of images of the bacteria because the focal point heat between the resin and the cellular structure of the bacteria. The focal point heat would make the proteins to be more closely packed configurations. These arrangements would also contribute to the contrast of the images of the bacteria. These effects can be observed clearly in the bacteria by the SMTEM compared to the bacteria by the RMTEM because the SMTEM's polymerization was set at 60°C/20 hours compared to the RMTEM's polymerization at 75°C/2 hours and 95°C/2 hours. The above mentioned effects were the granularities of the ribonucleic and patchy plasma membrane (Figure 1). Even though we mentioned earlier that granularities of the ribonucleic and patchy plasma membrane were damages caused by the OT, but we also believed that, the polymerization's focal heat point had also contributed to these damages. The reason would be that damages that were seen are more centralized in the inner core of the bacteria because the inner core of the bacteria would trap more heat compared to outer surface of the bacteria (and the outer membrane did not show any damages at all).

However, the gradual polymerization as in the RMTEM did not have these effects of focal point heat as the bacteria by the RMTEM had fine strand of ribonucleic and more comprehensive plasma membrane. Another feature would be the nucleotides were more prominent in the bacteria by the RMTEM compared to the bacteria by the SMTEM. This finding of the focal point heat damages was supported by the work done by Cohen (1982); he reported that the 60°C/8 hours had caused some damages to the membrane of amoebic samples.

Other benefits of the RMTEM compared to the SMTEM would be the introduction of the phosphate buffer in the glutaraldehyde solution and acetone as the dehydrating agent. Even though these introductions did not contribute to the contrast of the images but it does made the wholesome effects on the bacteria. It was reported that sodium cacodylate contains approximately 30% arsenic in weight and cacodylate avoids the presence of extraneous phosphate that may interfere with cytochemical studies. The buffer is incompatible with UA because the uranyl salts are one of the main staining properties used in the TEM staining method. Cacodylate is not very efficient buffer in terms of stabilizing the pH of the fixatives. This buffer causes changes in the membrane permeability and redistribution of cellular materials along osmotic gradients. However, the phosphate buffer would not present any hazardous effects to the laboratory staffs. Phosphate buffers are physiological because they found in living systems. These buffers were non-toxic to cell grown in culture, like the bacteria in this study. Phosphate buffers stabilize the pH of the fixative more efficiently than any other buffers (Hayat, 2000).

The dehydrating agent that was used in the RMTEM was acetone and for the SMTEM was ethanol. The boiling point of acetone is 56.5°C. Therefore, residual acetone in the specimen during embedding at 75°C and 95°C for the RMTEM in the embedding oven, rapidly and completely evaporated. The boiling points of ethanol and propylene oxide are 78°C and 35°C, respectively. Consequently, residual ethanol in the specimen during overnight embedding at 60°C for the SMTEM in the oven; will not evaporated the residual of ethanol that easily compared to the acetone because of higher boiling point of the ethanol. Residual ethanol inhibits the speed of resin polymerization, resulting in a soft block. Furthermore, the propylene oxide with lower boiling point compared to acetone is categorized under explosives material. It was stated that the miscibility of acetone with resins is faster than that shown by ethanol and propylene oxide (Hayat 2000).

Some evidence is available that indicates that acetone, a relatively non-reactive solvent, causes less specimen shrinkage and extraction of lipids than does ethanol. Acetone also has the advantage of not being reactive with residual OT (Page & Huxley, 1963). In addition, the dielectric

constant of acetone (20) is lower than the ethanol (24), indicating that the dehydration of the bacteria will be better with acetone compare to the ethanol. That is no evidence has yet been found indicating that the chemical interaction between epoxy monomers and acetone. Acetone has a keto group rather than an epoxy group, expected to inhibit polymerization of epoxy monomers; by contrast, a reactive monoepoxide (such as propylene oxide) could inhibit polymerization by combining with reactive groups of epoxy monomers (Hayat 2000).

CONCLUSION

The RMTEM is therefore is a better technique compared to the SMTEM especially in the TEM molecular studies of *Staphylococcus aureus* ATCC 25923. It is able to reduce the contrast and confine the cell contents of the bacteria closer to acquired conditions. Further detailed studies must be done on the RMTEM because the technique did not focus each factor that contributes to contrast of image of the bacteria, such as the GA, OT, and polymerization individually.

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